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AN INVESTIGATION OF THE INFLUENCE OF ENDOTOXINS ON MYOPLASMIC A--ETC(U)
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AN INVESTIGATION OF THE INFLUENCE OF ENDOTOXINS ON MYOPLASMIC ATPMg AND SODIUM EFFLUX IN BARNACLE MUSCLE FIBERS.

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Contract N00014-77-C-0112

The original goal of the present work was to investigate the possibility that endotoxins may act by inactivating the adenine nucleotide translocase system. This approach however to the problem of how endotoxins act had to be abandoned in view of evidence that barnacle muscle fibers have large phosphagen reserves e.g. an ArP content of 25 mmoles/kg fiber water and also have a myoplasmic ATPMg concentration of 1.2 mM. It should be recalled however that experiments involving the injection of endotoxin e.g. endotoxin from Serrata maresc. (obtained from Novotny) showed that the addition of 450 µg to the myoplasm of fibers poisoned with ouabain does sometimes lead to a stimulatory response. The same is true of lipid A injection. A different approach viz injection of endotoxin following peak stimulation by acidification of a HCO_3^--containing medium was adopted. As shown in Fig. 1, this, too, resulted in a stimulatory response. Furthermore, injection of endotoxin following peak stimulation by injected GTP resulted in a stimulatory response (Fig. 2). Taken together, these results raised the possibility that endotoxin exerts an effect whenever the phosphorylation-dephosphorylation cycle is stepped-up.

Inorder to be able to use the barnacle muscle fiber as a toxicological model, it seemed important in the light of the working hypothesis, namely that endotoxin reduces cell ATP, to adopt and adapt the firefly method to monitoring

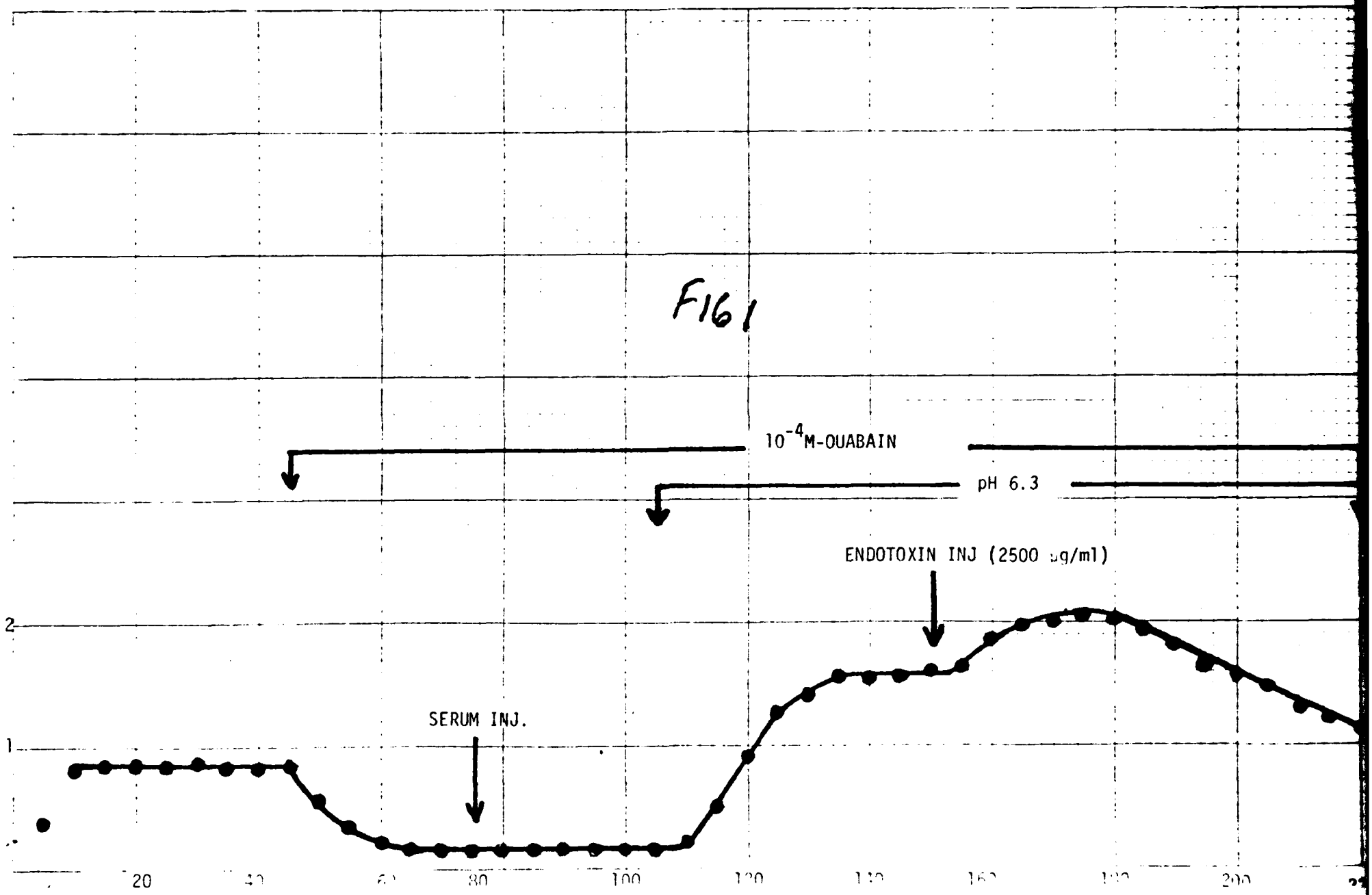

Fig 1

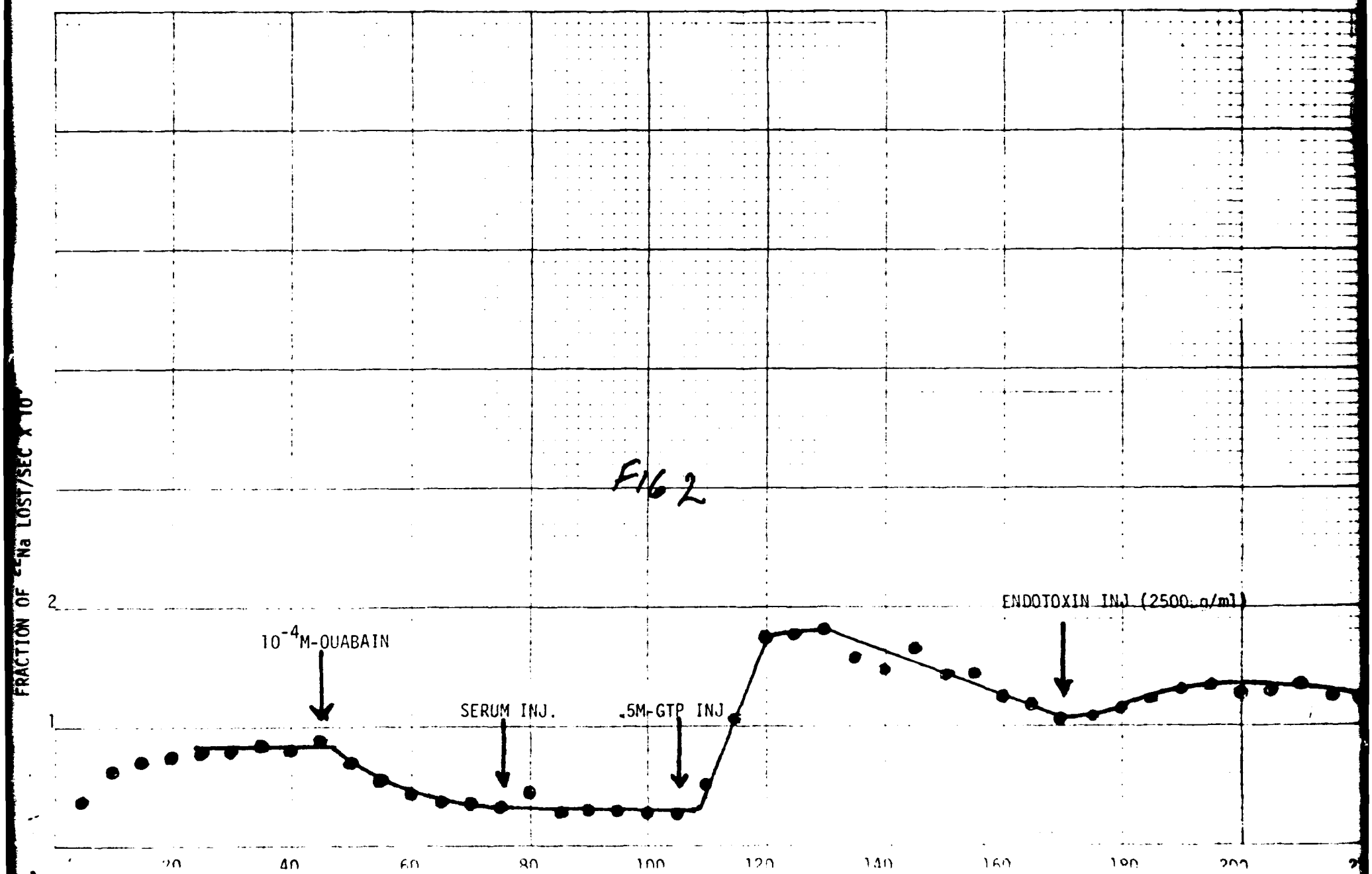
FRACTION OF ^{22}Na LOST/SEC X 10
2
1
10^{-4}M-OUABAIN
SERUM INJ.
.5M-GTP INJ
ENDOTOXIN INJ (2500 n/ml)
20
40
60
80
100
120
140
160
180
200

FIG 2

cytosolic ATP. This has been achieved (Bittar and Keh, 1980). Briefly, microinjection of DuPont luciferase-luciferin results in a flash the decay of which is initially rapid and then slow. Luminescence is quenched by injecting dehydroluciferin, Cl, PPi or raising pCO_2. By contrast, injection of CoA increases resting luminescence. These characteristics of the light reaction mixture in myoplasm resemble those of the cell-free system. Reinjection of the fiber with luciferase-luciferin 4 mins after the first injection results in a flash height that is practically equal to the first. External but not internal calibration based on the use of Kglutamate and a low Cl medium indicates that flash height is a linear function of the log of ATPMg over the 10^{-4} to 10^{-2}M range. Values obtained for myoplasmic ATPMg e.g. 1.2 ± 0.1 mM (n=24) have been repeatedly confirmed, not only with DuPont luciferase but also with a Packard preparation. However, experience additionally shows that Puget Sound specimens (B. nubilus) possess fibers which have the following myoplasmic ATPMg profile: a) ATPMg= 1.11±0.06 mM (n=32), b) ATPMg= 0.77±0.06 mM (n=26) and c) 0.48±0.04 mM (n=25). Samples of myoplasm obtained by aspiration, when assayed <u>in vitro</u>, are found to have an ATPMg concentration of 1.37 ± 0.14 mM (n=15) vs. 0.89 ± 0.15 mM (n=4) found <u>in situ</u> ($P < 0.05$). Addition of releasing reagent (SAI) to aspirate samples leads to a higher ATPMg value e.g. 2.02 mM. Further experiments along these lines are in progress.

Additionally, in collaboration with Dr. L.B. Hinshaw, plasma specimens (control and test) from dogs (and one baboon) in endotoxin shock were studied in two ways. One was to inject test plasma and see with the firefly method if myoplasmic ATP is altered. The other was to inject test plasma and see if the Na efflux in unpoisoned and ouabain-poisoned fibers is altered. The

results obtained are as follows:

Measurement of myoplasmic ATPMg

I.

Dog #1	Control	0.79 ± 0.05 mM, n=3	(P > 0.1)
	Test	0.67 ± 0.02 mM, n=3	
	Endotoxin*		
	Time: 3 hrs		
Baboon	Control	0.60 ± 0.05 mM, n=2	(P > 0.4)
	Test	0.72 ± 0.10 mM, n=2	
	E. coli**		
	Time: 4 hrs		
Dog #2	Control	0.68 ± 0.07 mM, n=4	(P > 0.5)
	Test	0.62 ± 0.05 mM, n=4	
	Endotoxin		
	Time: 3 hrs		
Dog #3	Control	0.65 ± 0.10 mM, n=3	(P > 0.5)
	Test	0.65 ± 0.13 mM, n=3	
	Endotoxin		
	Time: 2 1/2 hrs		

NB The protocol adopted in the above experiments involved the injection of the control or test plasma followed by firefly. Calibration was done using the standard 120 mM - Kglutamate - low Cl medium (Bittar and Keh, 1980).

* Endotoxin = 3 mg E. coli endotoxin/Kg

= ≈ LD_{100} Difco B_5 strain

** E. coli = ≈ 2×10^{10} organisms/kg

= ≈ LD_{100} Pathogenic E. coli B_7

II.		% stimulation	% inhibition	n	ATPMg (mM)
Dog #4	Control		7.2%	3	1.50±0.59
	Test		6.3%	2	1.29±1.0
	Endotoxin* Time: 2 1/2 hrs.				
Dog #5	Control	2.2%		2	0.94·0.0
	Test	12.2%		2	1.23±0.37
	Endotoxin* Time: 3 hrs.				
Dog #6	Control		10.0%	2	1.21±0.55
	Test		5.7%	2	0.94±0.53
	Endotoxin* Time: 3 hrs.				

NB Control values of ATP = 1.19 ± 0.23 mM, n=14. The protocol adopted in the above experiments involved the injection of firefly followed by control or test plasma, and then firefly again. In this manner flash heights were compared.

Conclusion: (a) Neither control plasma nor test plasma interferes with the myoplasmic light reaction mixture.

(b) Neither control plasma nor test plasma alters the myoplasmic ATPMg level in situ.

Measurement of Na efflux before and after injecting test plasma in unpoisoned and ouabain-poisoned fibers.

These experiments were run by injecting control plasma, followed by test plasma. The fibers employed were dissected from the same barnacle specimen and divided into 2 batches, one unpoisoned while the other poisoned with 10^{-4}M-ouabain.

The results indicate that poisoned fibers show a small biphasic response to injected test plasma as illustrated in Figs. 3 and 4.

Control plasma can be seen to produce a small transitory stimulation. It is therefore inferred that the transitory rise following injection of test plasma is not due to the presence of endotoxin but that the small inhibitory effect may well be related to its presence. A small response is to be expected in view of dilution by the myoplasm of the causal agent(s).

Comments: It is quite clear that the barnacle muscle fiber can be used as a model for studies of this type. However, signal progress is not possible unless maneuvers leading to inactivation of the phosphorginine transferase system are found.

PUBLICATIONS

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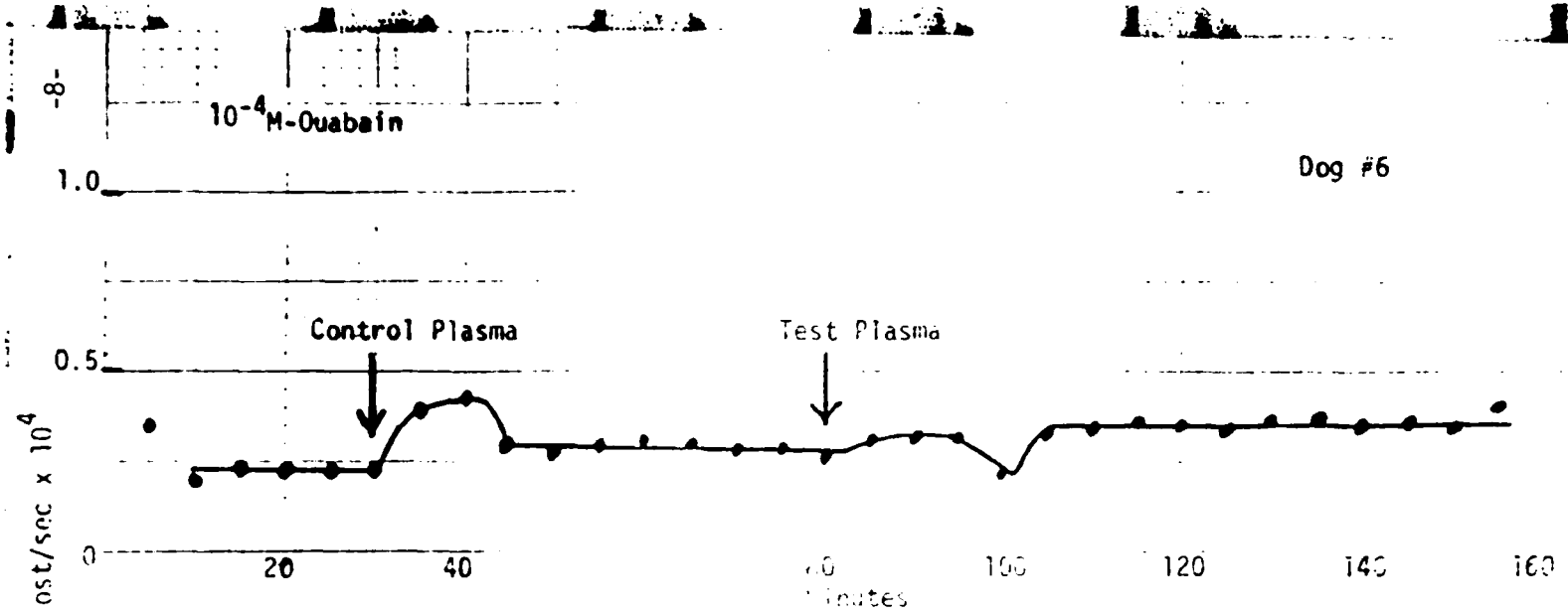
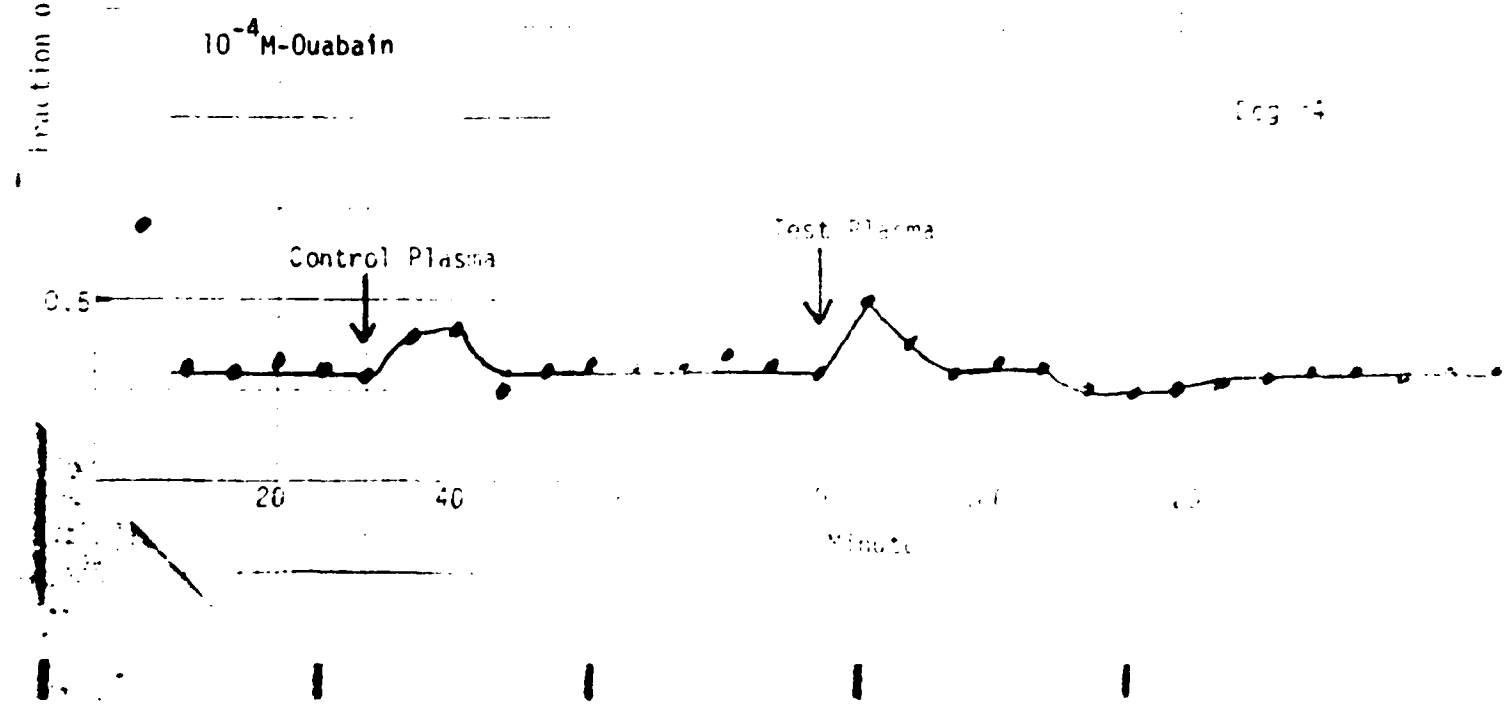

-8-
10^{-4}M-Ouabain
1.0
Dog #6
Control Plasma
Test Plasma
0.5
0
20
40
120
140
160
Minutes
Efflux of ^{22}Na lost/sec x 10^4

F16 3


10^{-4}M-Ouabain
Control Plasma
Test Plasma
0.5
20
40

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

The working hypotheses concerning the mechanism of action of endotoxin is that it reduces cytosolic ATP, possibly by inactivating adenine nucleotide translocase. Earlier work with endotoxin using the barnacle muscle fiber as a preparation led to evidence that endotoxin in low concentration, whether applied internally or externally, is without effect on the Na efflux in unpoisoned or ouabain-poisoned fibers. However, injection of endotoxin or lipid A led to a stimulatory response of the ouabain-insensitive Na efflux.

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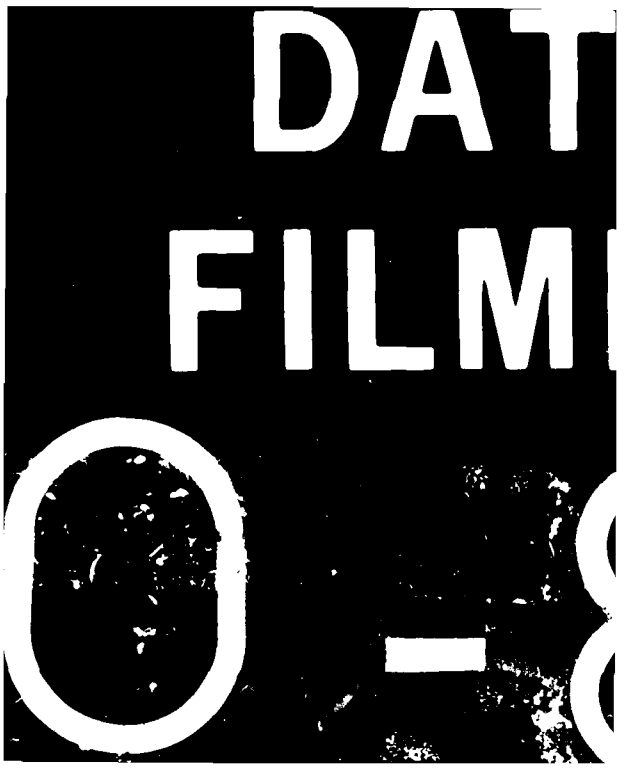
Inorder to test the hypothesis more directly, the firefly method of McElroy was adopted and adapted to measuring myoplasmic ATPMg. Considerable information about the kinetics and properties of the luminescence reaction in situ has been obtained (see Bittar and Keh, J. Physiology, April issue - 1980). More recently, efforts have been devoted to discovering maneuvers that would lead to a fall in myoplasmic ATPMg, more particularly maneuvers involving the glycolytic and oxidative phosphorylation pathways, as well as the arginine kinase pathway. The results obtained thus far have revealed that barnacle fibers have a large phosphagen reserve. This has been confirmed by ^{31}P-NMR. Hence the inference was drawn that unless the arginine phosphotransferase system can be blocked, it will be next to impossible to test the validity of the ATP hypothesis.

Experiments with plasma from dogs (and one baboon) in endotoxin shock have been done. These show a discrete biphasic response of the ouabain-insensitive Na efflux to injected plasma. Characteristically, a stimulation is followed by a transitory inhibition. Since stimulation is also observed with control plasma, it is deduced that the transitory inhibitory effect is genuine but not dramatic in size. It is not large probably because of the problem of dilution of the injectate by the myoplasm (this being roughly 100-fold).

The effect of injected plasma (from dogs in endotoxin shock) on myoplasmic ATPMg was investigated with the firefly method. The results indicate no alteration in the prevailing ATPMg level. This implies that the test plasma fails to interfere with the luminescence reaction itself. It also implies that if endotoxin (or other offending agent) reduces cytosolic ATP, mechanisms of ATP regeneration come into play almost instantaneously. On the other hand, it could be argued that neither endotoxin nor the plasma offending agent being sought act by reducing cytosolic ATP.

Insofar as studies with homopolymers e.g. polyinosinic acids, are concerned, these have not been undertaken.

Madison, February 28, 1980 E. Edward Bittar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